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Honeybee apisimin and plant arabinogalactans in honey costimulate monocytes

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ABSTRACT

Here we determined whether immunostimulatory plant-derived arabinogalactan proteins (AGPs) and the honeybee-derived protein apisimin are present in varieties of New Zealand honey. Apisimin is a protein of unknown function secreted from the glands of honeybees into Royal Jelly, forming a complex with apalbumin1 capable of stimulating lymphocyte proliferation. AGPs were abundant in kanuka honey with lesser amounts in manuka, kowhai and clover honeys, but absent from Royal Jelly. Apisimin was present in all honeys, as well as Royal Jelly. We report that apisimin shares with honey AGPs the ability to stimulate the release of TNF-α from blood monocytes. Further, it synergizes with AGPs to enhance the release of TNF-α, via a mechanism not involving the formation of a complex with AGPs. In summary, this study provides evidence that AGPs and apisimin are commonly present in different floral varieties of honey, and hence contribute to their immunostimulatory properties.

1. Introduction

Honey has been used since ancient times not only as a food product, but also as a wound dressing (Zumla & Lulat, 1989). Its use in modern day woundcare is widely studied and debated (Bardy, Slevin, Mais, & Molassiotis, 2008; Biglari et al., 2013; Bischofberger et al., 2013; Jull, Rodgers, & Walker, 2008; Jull, Walker, & Deshpande, 2013; Kamaratos et al., 2014; Lee, Sinno, & Khachemoune, 2011; Molan, 2006). There is an intensive effort underway to identify the bioactive components of honey, and their properties, in order to better inform the industry and the consumer of each honey product's potential properties, and to improve woundcare formulations. The properties of different honeys vary greatly, due in part to the particular floral nectars that are gathered by honeybees, and the different proteins secreted from the cephalic glands of honeybees (Rossano et al., 2012; Wang & Li, 2011). Unfractionated honey is immunostimulatory, being able to stimulate the release of TNF-α from monocytes/macrophages (Gannabathula et al., 2012; Raynaud et al., 2013; Tonks, Cooper, Price, Molan, & Jones, 2001; Tonks et al., 2003), with different honeys varying in their ability to stimulate TNF-α release (Gannabathula et al., 2012). TNF-α produced by monocytes/macrophages within a wound has been shown to promote the degradation and remodeling of the extracellular matrix, leading to accelerated healing and improved wound disruption strength (Fu, Tian, Hsu, Wang, & Sheng, 1996; Mooney, O'Reilly, & Gamelli, 1990). Conversely inhibition of TNF-α leads to decreased wound strength. Honey also stimulates monocytes/macrophages to produce interleukin (IL)-6 and IL-1β that induce extracellular matrix synthesis by fibroblasts (Cooper, Jones, & Morris, 2005, chap. 10; Tonks et al., 2003).

The identity of the key immunostimulatory components of honey is controversial. Tonks et al. isolated a polymyxin B-insensitive component of 5.8 kDa from manuka honey, which stimulated a human monocytoid cell line to release TNF-α via a Toll-like receptor 4-mediated mechanism (Tonks et al., 2007). Apalbumins (Apa) 1 and 2 of 55 kDa stimulated the release of TNF-α from macrophages and keratinocytes (Majtan, Kovacova, Bilikova, & Simuth, 2006; Majtan, Kumar, Majtan, Walls, & Klaudiny, 2010; Simúth, Bíliková, Kovácová, Kuzmová, & Schroder, 2004). However, Timm et al. reported that the immunostimulatory activity of manuka honey is explained solely by its endotoxin content (Timm,

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Bartelt, & Hansen, 2008). Conversely, we and Raynaud et al. reported that the trace amount of lipopolysaccharide (LPS) in kanuka and thyme honeys could not, by itself, account for the stimulation of cytokine production by honey (Gannabathula et al., 2012; Raynaud et al., 2013). We recently reported that plant-derived arabinogalactan proteins (AGPs) in New Zealand kanuka honey stimulate monocytes to release TNF-α (Gannabathula et al., 2012). The AGPs were sensitive to inhibition by polymyxin B, and hence appear to mimic some of the properties of LPS. Apisimin is a 5.5 kDa serine and valine-rich peptide that is secreted from the hypopharyngeal and mandibular glands of honeybees into Royal Jelly (Bíliková et al., 2002), forming a complex with Apa1 that is capable of stimulating the proliferation of lymphocytes (Tamura et al., 2009).

The immunostimulatory properties of honey are seemingly more complex as honey also stimulates the expression of anti-inflammatory products from monocytes/macrophages, including prostaglandin E2 (Raynaud et al., 2013), which improves wound healing (Talwar, Moyana, Bharadwaj, & Tan, 1996), and hastens wound closure (Savla, Appel, Sporn, & Waters, 2001), and the anti-inflammatory cytokines anti-inflammatory cytokines IL-10, IL-1ra, and transforming growth factor (TGF-β) (Bean, 2012). The flavonoid chrysin present in honey and propolis has potent anti-inflammatory properties (Ha, Moon, & Kim, 2010). Fir honeydew honey contains the flavonoids apigenin and kaempferol, which inhibited TNF-α-induced production of matrix metalloproteinase (MMP)-9 from keratinocytes (Majtan et al., 2013). In contrast, Acacia honey stimulated keratinocytes to release MMP9, together with TNF-α, IL-1β and TGF-β (Majtan et al., 2010). Manuka honey, and to a lesser extent kanuka and rewarewa contain the anti-microbial methyl glyoxal, which modifies the apalbumins, endowing them with an ability to inhibit phagocytosis by macrophages (Bean, 2012). Thus, the immunoregulatory properties of honey are complex, being dictated by the levels of individual pro-and anti-inflammatory components.

In this study, we sought to determine whether AGPs and apisimin are present in kanuka honey and other floral varieties of New Zealand honey, and to determine their contribution to the immunostimulatory properties of kanuka honey.

2. Materials and methods

2.1. General

New Zealand honey samples, namely young manuka (*Leptospermum scoparium*) honey (INTPH-01), young kanuka (*Kunzea ericoides*) honey (INTPH-15), clover (*Trifolium spp.*) honey (INTPH-20), and Royal Jelly (*Apis mellifera*) were selected and supplied by Comvita New Zealand Ltd, Te Puke. The young honeys were 6–18 months old. Artificial honey was prepared by dissolving 192 mg fructose, 180 mg glucose and 4 mg sucrose (Sigma Aldrich, New Zealand) in 10 ml of deionized water, which is equivalent to 4% of honey solution. LPS isolated from a culture of *Escherichia coli* 0127:B8 was purchased from Sigma Aldrich. The Yariv reagent was obtained from Callaghan Innovation, New Zealand, and an N-terminally biotinylated apisimin peptide (KTSISVKGESNVDVVSQINSLVSSIVSGANVSAVLLAQTLVNILQILIDANVFA) was synthesized and supplied by Peptide 2.0, USA. Mouse anti-human TNF-α antibodies were purchased from BD Biosciences, Auckland, New Zealand. A polyclonal mouse antiserum raised against synthetic apisimin was custom-produced by Promab Biotechnologies, Inc., CA. Fresh human blood monocytes were obtained from the buffy coats of venous blood fractionated by centrifugation on Ficoll gradients. Blood was obtained from healthy adult subjects by informed consent according to approval 2010/462 from the University of Auckland Human Participants Ethics Committee.

2.2. Radial agar diffusion assay

A radial agar diffusion assay to measure the AGP content of honey was developed based on a protocol previously described by van Holst and Clarke (1985). The assay is based on the fact that arabinogalactans are bound and precipitated in solution by the β-galactosyl Yariv reagent. Molten agarose gel (1%) containing 0.15 M NaCl, 0.02% sodium azide and 0.002% of the β-galactosyl Yariv reagent was poured into a 80 mm petri dish. The gel was allowed to set, and then wells were punched. Sample solutions (15 μl) prepared in 0.15 M NaCl buffer solution containing 0.02% sodium azide were pipetted into the wells. Buffer was used as a negative control, and Gum arabic (Sigma Aldrich) was used as a positive control, respectively. Petri dishes were sealed and incubated in the dark at room temperature for 2–4 days until a precipitin halo developed.

2.3. Rocket gel electrophoresis assay

AGPs in honey were quantified by rocket gel electrophoresis using Gum arabic as a standard according to a previously described method (Komalavilas, Zhu, & Nothnagel, 1991; Wiśniewska & Majewska-Sawka, 2007). Molten agarose (1%) containing 25 mM Tris, 200 mM glycine, pH 8.4, and 15 μM Yariv reagent was poured into plates to give a gel of 1.5 mm thickness. Wells were punched in the gel, and loaded with the honey samples and controls. Electrophoresis was carried out in Tris–glycine buffer (25 mM Tris, 200 mM glycine, pH 8.4) for 4–8 h at room temperature (RT) until the rockets were well developed. The concentrations of AGP in the samples were estimated in relation to the area of the precipitin peak formed with Gum arabic.

2.4. Crossed gel electrophoresis

AGPs purified from kanuka honey (Steinhorn, Sims, Carnachan, Carr, & Schlothauer, 2011) were compared with Gum arabic by two-dimensional crossed electrophoresis (Komalavilas et al., 1991; Wiśniewska & Majewska-Sawka, 2007). AGPs (5 μg) were initially separated in a 1% agarose gel as described for rocket electrophoresis, and then electrophoresed in a second direction in a 1% agarose gel supplemented with 15 μg/ml Yariv reagent, which precipitated the AGPs as they moved towards the anode.

2.5. Polyacrylamide gel electrophoresis

Polyacrylamide gels were prepared as described in Current Protocols in Molecular Biology (Ausubel, 1999). Synthetic apisimin was analysed on a non-denaturing 15% polyacrylamide gel, and on a denaturing 16% polyacrylamide Tricine SDS-gel prepared as described previously (Schägger, Aquila, & Von Jagow, 1988; Schägger & Von Jagow, 1987). The protein was visualised by staining with Coomassie blue G-250.

2.6. Ligand blot assay

A ligand blot assay was established to determine whether apisimin interacts with honey AGPs. Samples of 2 μl of each of synthetic apisimin (1 mg/ml), honeys (100 mg/ml), purified kanuka honey AGPs (1 mg/ml), bovine serum albumin (BSA) (1 mg/ml), and insulin (1 mg/ml) were spotted onto a nitrocellulose membrane. The membrane was blocked for 1 h with 5% skim milk powder in Tris-buffered saline containing 1% Tween 20 (TBS-T). The membrane was washed thrice for 5 min with TBS-T and incubated with 0.1–10 μg/ml synthetic biotinylated apisimin peptide for 1 h at RT. The membrane was washed thrice for 5 min with TBS-T, incubated with horseradish peroxidase (HRP)-conjugated

streptavidin for 30 min at RT, and HRP activity detected by enhanced chemiluminescence.

2.7. Western blot analysis

Samples separated by SDS–PAGE on Tricine gels were electroblotted onto nitrocellulose membrane. The membranes were blocked with TBS-Tween buffer containing 5% non-fat milk for 1 h at RT, and incubated overnight with a polyclonal mouse antiserum raised against synthetic apisimin, diluted 1:50 in TBS-Tween buffer containing 5% non-fat milk. They were washed and incubated for 2 h in the same buffer containing HRP-conjugated anti-mouse IgG at a dilution of 1:5000, and HRP activity detected by enhanced chemiluminescence.

2.8. TNF-α ELISA

TNF-α concentration was measured by ELISA as described previously (Gannabathula et al., 2012). Briefly, a mouse anti-human TNF-α mAb used as the capture antibody was coated onto ELISA plates at 2 μg/ml, and 100 μl of culture supernatant was placed into the wells. After washing, a biotinylated mouse anti-human TNF-α antibody at 2 μg/ml was added to detect the captured TNF-α. Immunoreactivity was detected with streptavidin-horseradish peroxidase, and developed with tetramethylbenzidine (TMB) Substrate Reagent (BD Biosciences). LPS (100 ng/ml) was included as a standard, against which the results obtained with the honey samples were compared.

2.9. Statistical analysis

The graph packages used were Graph Pad (Prism) software and Microsoft Excel. Results were expressed as means ± SD, and the overall differences between group means were analysed using one-way ANOVA. Multiple comparisons were carried out using Tukey's Test.

3. Results and discussion

3.1. Agar diffusion and rocket gel electrophoresis assays to establish the levels of AGPs in honeys

An agar diffusion assay in which the Yariv reagent was added to the agar was employed to establish the level of AGPs in kanuka honey. Samples (15 μl) of Gum arabic AGPs (500 μg/ml), Gum arabic (100 μg/ml), purified kanuka honey AGPs (500 μg/ml), kanuka honey (undiluted), and LPS (100 ng/ml) were loaded into the wells and allowed to diffuse. Gum arabic and purified kanuka honey AGPs produced bright orange haloes that were formed by precipitation of the AGPs with the Yariv reagent (Fig. 1A). Crude kanuka honey and Gum arabic each produced fainter precipitin haloes with smaller diameters. The diameter and intensity of the halo

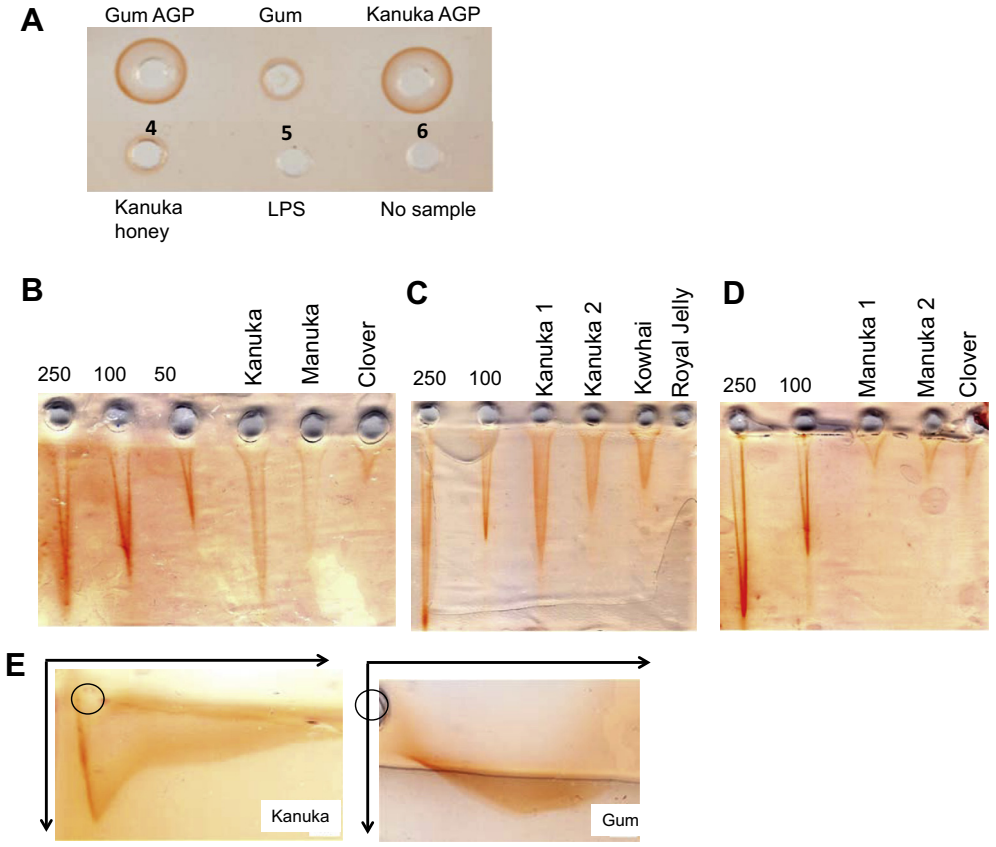

Fig. 1. Analysis of AGPs in different honey varieties. (A) Single radial gel diffusion assay using the Yariv agent to measure the concentrations of AGPs in kanuka honey. The samples analysed included Gum AGP (500 μg/ml), Gum arabic (100 μg/ml), kanuka honey AGP (500 μg/ml), kanuka honey (20 μl), LPS (100 ng/ml), and no sample. Precipitin haloes are naturally coloured orange due to the brown-orange colour of the Yariv agent. (B–D) Rocket electrophoresis using the Yariv agent to measure the concentrations of AGPs in different honey varieties. (B) The samples analysed included 20 μl of undiluted kanuka, manuka, and clover honeys. Gum arabic was included as a standard at 250, 100, and 50 μg/ml, as indicated. (C) The samples included 20 μl of two different sources of kanuka honey, kowhai honey, and Royal Jelly. Gum arabic was included as a standard at 250, 100 μg/ml, as indicated. (D) The samples included 20 μl of two different sources of manuka honey, and clover honey. Gum arabic was included as a standard at 250, 100 μg/ml, as indicated. (E) Crossed electrophoresis of Gum arabic and kanuka honey AGP. Five micrograms of each sample was electrophoresed in the first dimension (left to right) in ordinary agarose, and then in the second dimension (top to bottom) in gels containing the β-glucosyl Yariv reagent (15 μg/ml). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was proportional to the amount of AGP in the well. Thus, identical concentrations of gum arabic and honey AGPs gave similar sized haloes. Lipopolysaccharide (LPS) did not produce a halo, establishing that it does not react with the Yariv reagent.

Rocket gel electrophoresis in which the Yariv reagent was added to the agar was employed to establish the presence and levels of AGPs in a variety of different honey types. There was 200, 60, and 25 μg/ml of AGPs in kanuka, manuka and clover honeys, respectively (Fig. 1B). Thus, kanuka honey is rich in AGPs, whereas clover honey has a low level of AGPs. In another experiment, two sources of kanuka honey were shown to differ in the level of AGPs, having levels of 180 and 90 μg/ml, respectively (Fig. 1C). Kowhai honey had intermediary levels of AGPs of 60 μg/ml, whereas AGPs were apparently completely absent from Royal Jelly. In a third experiment, two different sources of manuka honey from that shown in Fig. 1B were found to have low levels (35–40 μg/ml) of AGPs, comparable to that of clover honey (Fig. 1D). Crossed-electrophoresis profiles indicated that kanuka honey AGPs were different from Gum arabic AGPs (Fig. 1E). Gum arabic AGPs gave a single peak in accord with the fact that the major molecular fraction accounting for ~88% of the total mass of Gum arabic is an arabinogalactan peptide with a molecular weight of 286 kDa (Sanchez et al., 2008). Kanuka honey AGPs gave a major and a minor peak, where only the minor peak resembled that formed by Gum arabic.

3.2. Identification of apisimin in honey

Here we sought to determine whether the immunostimulatory protein apisimin was present in honey, given that it is secreted from the hypopharyngeal and mandibular glands of nurse and forager honeybees, and has been reported to form a complex with the Royal Jelly protein Apa1 (Tamura et al., 2009). Synthetic apisimin was first resolved by non-denaturing and denaturing polyacrylamide gel electrophoresis to determine its migration profile on SDS–PAGE, as it has been reported to form a self-associated complex of 30 kDa (Bíliková et al., 2002). It is detectable with Coomassie blue staining despite the fact that it contains only one of the five amino acids required to bind this stain. Synthetic apisimin migrated as a high molecular weight protein of around 30 kDa on a non-denaturing polyacrylamide gel, despite the fact that it is only a 54 amino acid residue peptide with a predicted molecular weight of 5.8 kDa (Fig. 2A). In marked contrast, it migrated as a homogeneous low molecular weight band of less than 6 kDa on a denaturing Tricine-based SDS–polyacrylamide gel (Fig. 2B). Thus apisimin appears to form oligomers as reported by Bilikova et al. The apisimin band on the denaturing gel was broad, presumably because the peptide forms oligomers containing different numbers of the apisimin peptide. Samples of kanuka honey and Royal Jelly were resolved by SDS–PAGE on 15% polyacrylamide gels, and the proteins were stained with Coomassie blue. The major apalbumin bands at ~55 kDa were clearly evident in the kanuka honey and Royal Jelly samples, but in addition both samples contained a low molecular band similar in size to apisimin (Fig. 2C).

A commercially prepared mouse anti-apisimin polyclonal antiserum was employed to determine whether the low molecular weight band seen in kanuka honey and Royal Jelly samples was apisimin. Samples of kanuka, manuka and clover honeys, and Royal Jelly were resolved on a denaturing 15% polyacrylamide gel and subjected to Western blot analysis with the anti-apisimin antibody. The antibody detected a major low molecular band of ~5 kDa protein in the 3 honeys and Royal Jelly, which was identical in size to chemically synthesized apisimin (Fig. 2D). Trace amounts of slightly higher molecular weight forms were evident, which may represent small oligomers that were not completely dissociated. Kanuka honey appeared to contain the highest levels of apisimin amongst the honeys. Western blot analysis using synthetic apisimin as a standard revealed that apisimin was present in kanuka honey at a concentration of 100 μg/ml.

3.3. Apisimin stimulates the release of TNF-α from blood monocytes

Increasing concentrations of apisimin were tested for their ability to stimulate blood monocytes to release TNF-α. For comparison monocytes were also stimulated with increasing concentrations of kanuka honey AGPs that had been purified with the Yariv reagent. Apisimin stimulated the release of TNF-α in a concentration-dependent fashion, with a similar activity to that of the kanuka honey AGPs (Fig. 3A). The response was linear for both apisimin

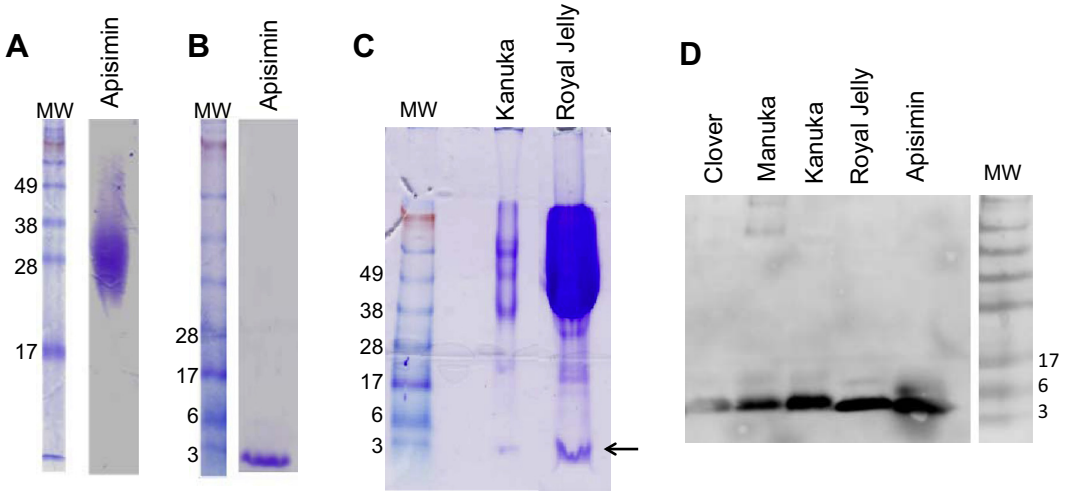

Fig. 2. Analysis of commercially synthesized and endogenous apisimin. (A) Non-denaturing polyacrylamide gel analysis of synthetic apisimin (15 μg). MW, molecular weight protein standards (Sigma). The sizes of the molecular weight makers are given in kDa in the left-hand margins. (B) Denaturing Tris-Tricine SDS–polyacrylamide gel analysis of synthetic apisimin (15 μg). MW, molecular weight protein standards (Sigma). (C) Both honey and Royal Jelly contain a low molecular weight protein similar in size to apisimin. Samples of kanuka honey (15 mg), and Royal Jelly (25 μg) were electrophoresed on 6% polyacrylamide Tricine SDS-gels. MW, molecular weight protein standards (Sigma). The position of the putative apisimin band is marked with an arrow. (D) Western blot analysis detects apisimin in New Zealand honeys and Royal Jelly. Aliquots of 15 mg of clover, manuka, and kanuka honey, and 150 μg Royal Jelly, and 25 μg of synthetic apisimin were resolved on a Tris-Tricine polyacrylamide gel, and screened by Western blot analysis using a polyclonal mouse antiserum raised against synthetic apisimin. Immunoreactivity was detected with HRP-conjugated anti-mouse IgG, followed by enhanced chemiluminescence. The sizes of the molecular weight makers are given in kDa in the right-hand margin.

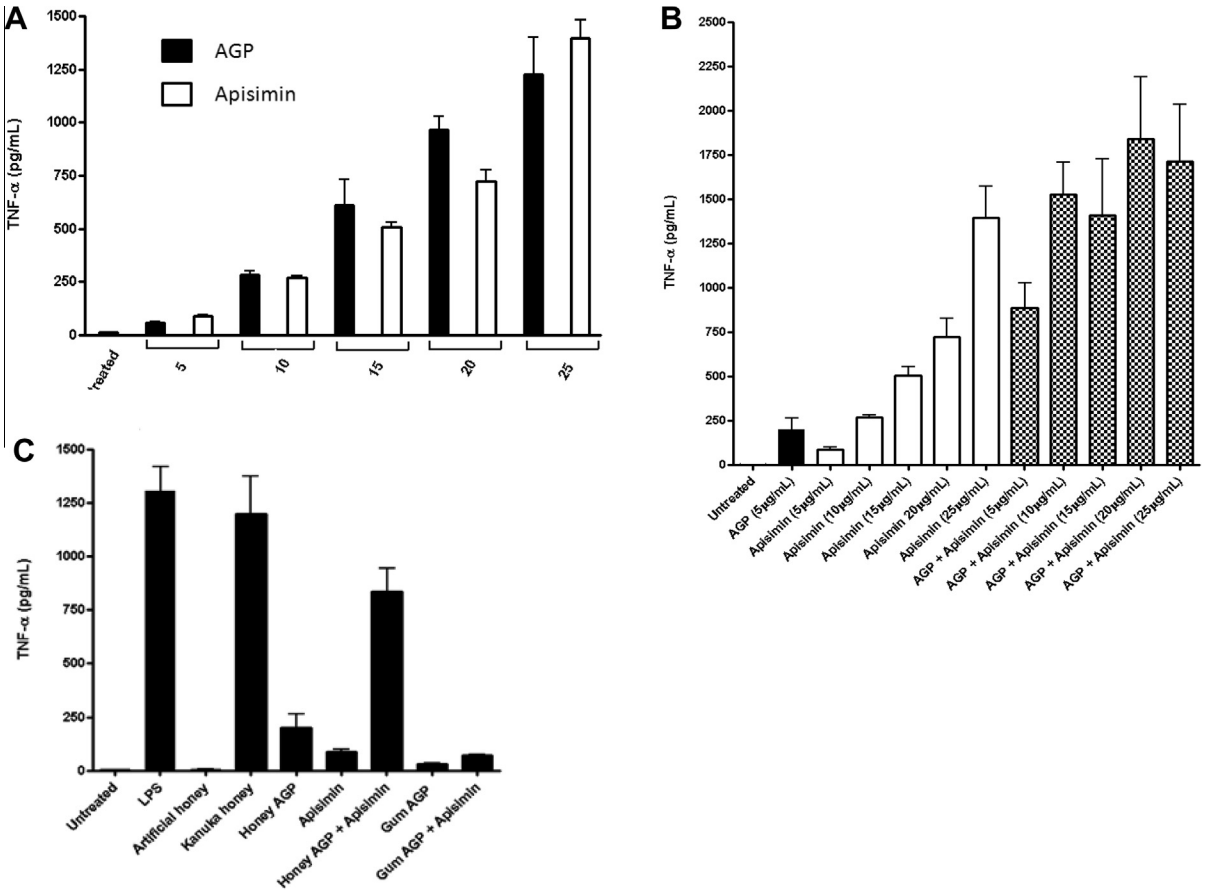

Fig. 3. AGPs and apisimin synergize to stimulate the release of TNF-α from blood monocytes. (A) Peripheral blood monocytes were incubated with increasing concentrations (5–25 μg/ml) of purified kanuka honey AGPs and synthetic apisimin. TNF-α release was measured by ELISA and presented as mean + SD of four replicates. (B) Blood monocytes were incubated with 100 ng/ml LPS, 1% (w/v) kanuka honey, 5 μg/ml of AGP, 25 μg/ml apisimin, and 5 μg/ml of AGP in combination with 25 μg/ml apisimin AGP. (C) Blood monocytes were left untreated or incubated with 5 μg/ml of AGP alone and in the presence of increasing concentrations of apisimin (5–25 μg/ml). Control monocytes were stimulated with apisimin (5–25 μg/ml) in the absence of AGP. TNF-α release was measured by ELISA and presented as mean + SD of four replicates.

($r2$ = 0.8969) and AGP ($r2$ = 0.9973) over the tested concentrations of 5–25 μg/ml.

3.4. Apisimin synergizes with kanuka honey AGPs to stimulate the release of TNF-α from blood monocytes

Apisimin and kanuka honey AGPs both stimulate the release of TNF-α from blood monocytes. There was the possibility that they might synergize with one another, given that they both induce the expression of TNF-α (Gannabathula et al., 2012). To address this notion, blood monocytes were stimulated with 5 μg/ml of synthetic apisimin alone or in combination with 5 μg/ml of kanuka honey AGP. Samples of kanuka honey AGP, artificial honey and LPS were included for comparison. Apisimin and kanuka honey AGP again stimulated the release of TNF-α, at low levels compared with 0.1 μg/ml of LPS (Fig. 3B). Surprisingly, apisimin strongly synergized with kanuka honey AGP to increase TNF-α release, which was comparable to the effect achieved with 10 mg/ml (1%) of kanuka honey. Thus, individual stimulation with 5 μg/ml of AGP and 5 μg/ml of apisimin caused the release of 199 and 86.6 pg/ml of TNF-α, respectively, whereas the combination caused the release of 840 pg/ml of TNF-α, effectively a threefold increase in TNF-α release. In contrast, apisimin did not synergize with Gum arabic, which had negligible activity comparable to that of artificial honey. To explore the relationship between apisimin and AGP further, increasing concentrations of apisimin (5–25 μg/ml) were used to stimulate blood monocytes in the presence of a constant concentration of kanuka honey AGP (5 μg/ml) (Fig. 3C). The synergistic effects of apisimin with AGP were also concentration-dependent, where the strongest synergy was observed with AGP in combination with low levels of apisimin. Apisimin showed strong stimulation by itself at high concentrations.

3.5. Apisimin does not form a complex with honey AGPs

It was plausible that apisimin might form a multivalent complex with honey AGPs, as previously described for the apalbumins (Tamura et al., 2009), that could explain its synergistic properties. A ligand-binding assay was established to test this notion in which apisimin, kanuka honey AGPs, kanuka honey, manuka honey, clover honey, and the control proteins bovine serum albumin and insulin were spotted onto a nitrocellulose membrane, and screened for their ability to bind biotinylated apisimin. Apisimin bound to itself in accord with its ability to form oligomers (Fig. 4). It also bound to each of the honey samples, in agreement with the finding that each honey sample contains apisimin and apalbumins. In contrast, apisimin did not bind to purified kanuka honey AGPs, indicating it does not form a complex with honey AGPs.

In summary, this study has revealed that all honey samples tested, including kanuka, manuka, kowhai and clover honeys, contain AGPs. Kanuka honey was particularly rich in AGPs, whereas manuka, kowhai, and clover honeys by comparison contained only low concentrations of AGPs. Surprisingly, Royal Jelly completely lacked AGPs. Crossed gel electrophoresis suggested that kanuka

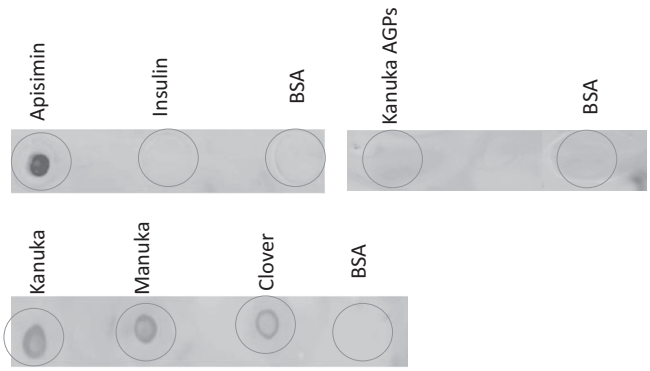

Fig. 4. Apisimin does not bind kanuka honey AGPs. Samples of 2 μl of each of synthetic apisimin (1 mg/ml), insulin (1 mg/ml), bovine serum albumin (BSA) (1 mg/ml), kanuka honey, manuka honey, clover honey, and purified kanuka honey AGPs were spotted onto a nitrocellulose membrane. The membrane was incubated with biotinylated apisimin, and binding of apisimin to spotted samples was detected with horseradish peroxidase (HRP)-conjugated streptavidin and enhanced chemiluminescence.

honey AGPs were more heterogeneous than those present in Gum arabic. We have similarly confirmed that apisimin is present in kanuka, manuka, and clover honeys, in addition to Royal Jelly. In a novel finding, it was discovered that apisimin shares with honey AGPs the ability to stimulate the release of TNF-α from blood monocytes. Further, it synergizes with AGPs to enhance the release of TNF-α, via a mechanism that does not involve forming a complex with AGPs. We have confirmed that apisimin self-associates to form oligomers that could play a role in its immunostimulatory ability. In a previous study we revealed that kanuka honey was more effective at inducing the release of TNF-α from monocytes (Gannabathula et al., 2012), which is in accord with the present finding that kanuka honey contains higher concentrations of AGPs and apisimin.

There is very little information in the published literature regarding the biological properties and role of apisimin. It was reported to be a 5.5 kDa protein component of Royal Jelly (Bíliková et al., 2002), which formed a complex with Apa1 that is capable of enhancing and sustaining the proliferation of the human lymphoid cell line Jurkat (Tamura et al., 2009). Tonks et al. reported an unidentified polymyxin B-insensitive component of 5.8 kDa from manuka honey, which stimulated a human monocytoid cell line to release TNF-α via a Toll-like receptor 4-mediated mechanism (Tonks et al., 2007). The 5.8 kDa appeared to bind to a larger molecular weight component (>30 kDa) that was largely responsible for the immunostimulatory activity of manuka honey. These latter properties are remarkably similar to those of apismin, raising the question of whether apsimin is the 5.8 kDa immunostimulatory component identified by Tonks et al. The results also suggest that the apalbumins may not be immunostimulatory as reported, but rather their activity is due to their association with apisimin. Tonks' brief analysis concluded that apisimin was unlikely to be a protein, but apisimin lacks tryptophan and tyrosine residues which render it silent at A280 nm. Monosaccharide component analysis revealed the presence of monosaccharides (Tonks et al., 2007), which is in accord with the extensive potential serine/threonine and asparagine sites within apisimin for N- and O-linked glycosylation, respectively. In a future study it will be important to determine whether natural apisimin is glycosylated, and whether glycosylation affects its immunostimulatory activity, and associations with other honey components.

This study, together with previous reports, highlights the fact that honey is a complex mixture of proinflammatory and anti-inflammatory bioactive components, which may differ depending on the particular floral nectars that are gathered by honeybees, and the products secreted by the honeybees themselves.

4. Conclusion

This study provides evidence that AGPs and apisimin are commonly present in different varieties of honey, and contribute to their immunostimulatory properties. The data to date indicates that the immune-stimulatory properties of honey arise from a synergism displayed by its various bioactive components, including AGPs, apisimin, apalbumins, and any contaminating LPS. Further studies are required to determine the specific quantities of these bioactive components in a broader range of honey varieties, their effects on health and well-being, and their potential application, including their contribution to the treatment of wounds.

Acknowledgements

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